A POX-LIKE DISEASE IN CYNOMOLGUS MONKEYS

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During the summer and fall of 1958 two outbreaks of a non-fatal pox-like disease in cynomolgus monkeys have been observed in the monkey colony in this institute. Both outbreaks occurred rather late after the monkeys had been received, *i.e.* 51 and 62 days after arrival and only a small percentage of the exposed animals showed signs of illness.

This paper presents observations on the epidemiological and clinical manifestations of the disease. The isolation of a virus from the diseased animals will also be described as well as some studies on the properties of the agent which in this paper will be referred to as monkey pox virus.

MATERIAL AND METHODS

Virus isolation: Material for egg inoculation was collected from the pustular lesions and diluted approximately 1: 10 in 0.004 M. McIlvain's buffer saline pH 7.2 (5). Material for tissue culture experiments was collected from the pustules by means of a cotton swab, which was immediately immersed into 2 ml of Bacto-tryptose "Difco". Both diluents contained Penicillin (100 U per ml) and Streptomycin (0.1 mg per ml). After clarification by low speed centrifugation the supernatants were used for inoculation onto the chorio-allantoic membrane of 11–12 day old Leghorn embryos and into tissue cultures of monkey kidney, human amnion and HeLa cells. A total of three virus strains was isolated, one during the first and two during the second outbreak. Most of the studies to be reported were carried out using the strain which was isolated first.

Vaccinia strain: Lot No. 1/50 III received from the smallpox vaccine department in this institute was used in some experiments in order to compare it with the monkey virus. This strain had been maintained for several years by serial cutaneous passages in rabbits and calves. Either the glycerinated third calf passage or a subsequent first passage of this material on the chorio-allantois was used.

Egg passages: The technique described by *Westwood et al.* (25) was employed. After inoculation the eggs were incubated for 48 hours at 37° C when vaccinia virus was used for infection and for 72 hours when the monkey virus was used.

Neutralization tests on the chorio-allantoic membrane: Undiluted serum was mixed with equal volumes of increasing dilutions of virus. After incubation for 2–3 hours at room temperature 0.1 ml amounts of the serum-virus mixtures were inoculated onto the chorio-allantoic membranes of groups of five 11–12 day old embryos. The membranes were harvested after 48–72 hours continued incubation and the reduction effected in the number of typical pocks was determined.

Tissue cultures: The techniques employed for the preparation of tissue cultures for infectivity titrations and for neutralization tests have been described in earlier publications (17, 20). An inoculum of 0.2 ml was used for tissue culture passages and the tubes were kept stationary at 35° C. During the first experiments they were examined daily for cytopathic changes but in later ones microscopic examination was carried out only every fourth or fifth day. Serial passages were usually made with 0.2 ml amounts of undiluted nutrient fluid harvested 5 days after inoculation.

Hemagglutination: The pattern method of *Salk* (22) was employed. The technique has been described in detail in previous papers (18, 20).

Complement-fixation tests were carried out according to the method of *Fulton & Dumbell* (11) as modified by *Svedmyr et al.* (24). Antigens were prepared from nutrient fluid of tissue cultures as well as from chorio-allantoic membranes infected with monkey-pox or vaccinia virus.

Rabbit immune sera: Hyperimmune sera against monkey pox and vaccinia viruses were prepared in rabbits by repeated intravenous injections of 0.5 to 2.0 ml amounts of 10 per cent suspensions of infected chorio-allantoic membranes. The animals received a total of 7 intravenous injections (on day 1, 9, 11, 16, 18, 23 and 25). They were bled 10 days after the last injection.

Another hyperimmune serum prepared by immunizing rabbits with calf-lymph vaccinia antigen which had been inactivated by heating at 70° C for 60 minutes was also used. In addition, an hyperimmune vaccinia rabbit serum kindly supplied by dr. F. O. MacCallum, England was used in some experiments.

A human hyperimmune serum was finally used in some egg-neutralization tests.

Diffusion-precipitation test was performed according to the method described by *Gispen* (12).

Electron microscopy: A prototype of the Phillips electron microscope type EM 100 B was used. Exposures were made at a magnification of 3000 × and photographically enlarged as desired.

The pus-like material from the pustular lesions of a monkey was removed by means of a capillary pipette, placed on formvar coated grids and allowed to dry. Following the method of *van Rooyen & Scott* (21) the grids were treated with 0.25 per cent crystalline trypsin solution for 4 hours at 37° C after which they had 2 brief washings in distilled water. Fixation was carried out in the vapours of a 1 per cent osmiumtetroxide solution for 15 minutes, and in order to minimize the risk of infection the grids were heated to 70° C for one hour (1) prior to shadowcasting with platinum. The egg adapted virus grown on chorio-allantoic membranes was purified for electron microscopy by means of differential centrifugation and precipitation with citric acid according to the method of *Henderson & McClean* (14). The final suspension was placed directly on formvar coated grids, dried and further treated as stated above with the exception that no digestion with trypsin was carried out.

EPIDEMIOLOGICAL DATA

Monkeys: This institute receives a continuous supply of monkeys which are used for polio vaccine production and research. Usually the monkey colony comprises several hundred animals, mostly Macacus Rhesus and Macacus Cynomolgus. The major part of the animals are kept in an animal house containing 15 cubicles which—according to their size—provide accomodation for 25 to 50 monkeys each. Occasionally, 100 to 150 monkeys are housed in a room (room B) in another building which is located about 200 yards from the main animal house, It is of interest that the first outbreak of the pox-like disease occurred among monkeys kept in the main building whereas the second outbreak some 4 months later began among animals housed in room B.

Outbreak No. 1: The first outbreak of the pox-like disease began on June 30th, 1958 in cynomolgus monkeys which had been received by

plane from Singapore on April 29th, 1958. On arrival this batch, consisting of 150 animals, was treated prophylactically with intramuscular injections of Penicillin and Streptomycin and subsequently distributed in 3 cubicles in the main animal house. Their general condition was satisfactory and only a few animals died during the following 2 weeks. From May 15th, however, an increase in mortality due to pneumococci (types 19 and 1) occurred. In order to compare the efficacy of antibiotics given by the parenteral and by the oral routes, respectively, the remaining animals (about one hundred—some animals having been sacrificed for experimental purposes during the preceeding weeks) were divided into two groups of 50 monkeys each, and housed in two different rooms. Group I was given intramuscular injections of Penicillin and Streptomycin for 3 days in succession while group II was fed food pellets to which aureomycin had been added. No difference was observed between the two groups but the mortality decreased gradually at almost the same rate in both groups and the animals appeared to have recovered.

On June 30th, however, a pox-like skin eruption was observed in one of the 32 monkeys remaining in group I. Two similar cases occurred on July 4th, and on July 7th two more animals in group I had vesiculopapular skin eruptions. A sixth and final case apparently in a later stage of the disease was noticed on July 14th. The outbreak thus lasted about two weeks involving a total of 6 animals (20 per cent). None of the remaining 26 animals showed any signs of the disease. Nor was any sign of the disease observed in group II or in the other monkeys housed in the main animal building.

Outbreak No. 2 occurred almost 4 months later in another shipment of 223 cynomolgus monkeys which had arrived by plane from Singapore on September 18th. Approximately, one hundred of these monkeys were kept in the main animal building while 120 animals were housed in room B. Excepting a few fatal pneumococcal infections, the health of all animals appeared good. On November 7th, however, 3 monkeys in room B were found to have skin lesions very similar to those seen during outbreak No. 1 four months earlier. During the next three days 8 additional cases were observed among the animals in this room. Clinical disease was thus observed in a total of 11 of the animals (approximately 10 per cent). However, when the monkeys were carefully examined one month later scattered healed lesions were observed in another 12 animals indicating that a total of 30 per cent of the animals had suffered from the disease.

At the time when outbreak No. 2 occurred in room B none of the monkeys of the same shipment living in the main animal building showed any signs of disease. But three weeks later, on Dec. 1st, one of these animals developed the dsease. A second case was observed 2 weeks later, on Dec. 12th.

Fig. 1.
Pox-disease. Cynomolgus monkey: Acute stage. Typical pustules.

CLINICAL MANIFESTATIONS

During both outbreaks no clinical signs of the disease were noted in the monkeys prior to the onset of the eruptive stage. At this time the clinical picture when fully developed was characterized by a generalized petecchial rash which rapidly developed into a maculopapular eruption. Judging from the scratched appearance of several of the lesions, particularly on the back of the animals, it seemed likely that the eruption was associated with some itching. The lesions were seen over the entire trunk and tail, on the face and the limbs of the animals, being particularly abundant and developed on the palms of the hands and the soles of the feet where the papules were also rather big (5×5 mm) and frequently umbilicated. The content of the papules became very thick and pus-like. Most of the papules appeared to be in the same stage of eruption although smaller variations were occasionally noticed (Fig. 1).

In addition to the fulminant cases just described a number of animals showed less extensive eruptions which were frequently most easily recognized on their tails.

The general health of the animals appeared quite unaffected at this stage as well as earlier and later in the disease. At autopsy no lesions were observed in the organs of the diseased animals.

Some of the monkeys were left alive and observed for a period of 2 to 4 months. In these animals the lesions were gradually covered with crusts which eventually fell off as healing progressed, leaving a distinct scar. (Figs. 2 and 3).

Fig. 2.

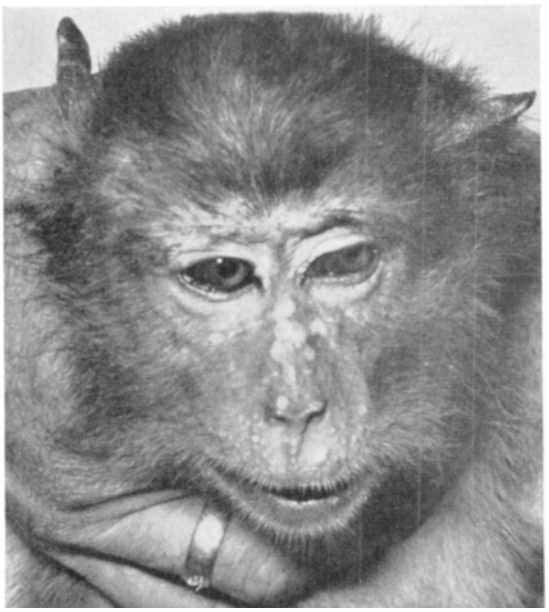

Fig. 3.

Figs. 2 and 3.
Pox-disease. Convalescent stage (approximately 4 weeks after skin eruption).
Lesions in healing and scars.

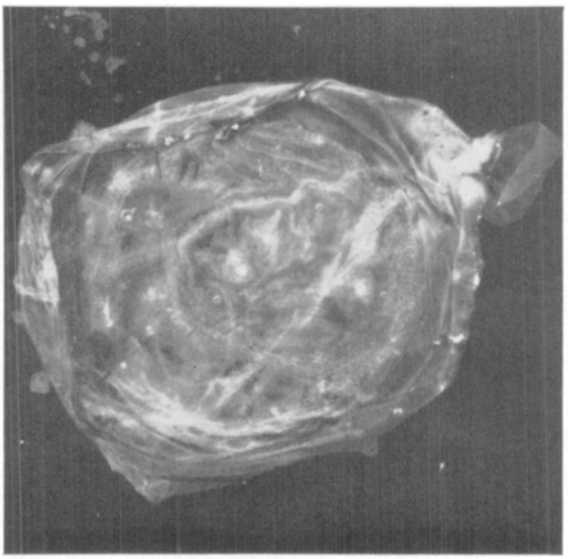

Fig. 4.

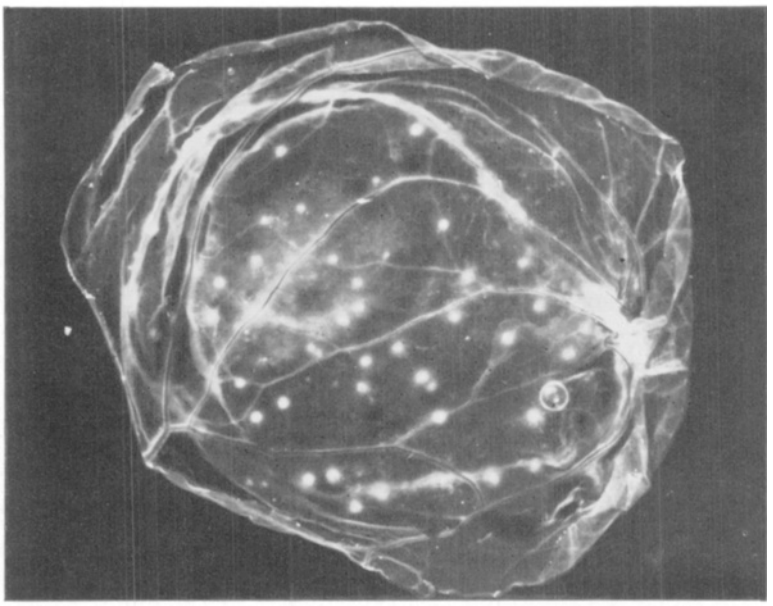

Fig. 5.

Figs. 4 and 5.
Monkey pox virus on the chorio-allantoic membrane.
Fig. 4. 1st egg passage: Oedematous reaction and few discrete lesions.
Fig. 5. 3rd egg passage 72 hours after inoculation: Typical discrete opaque foci.

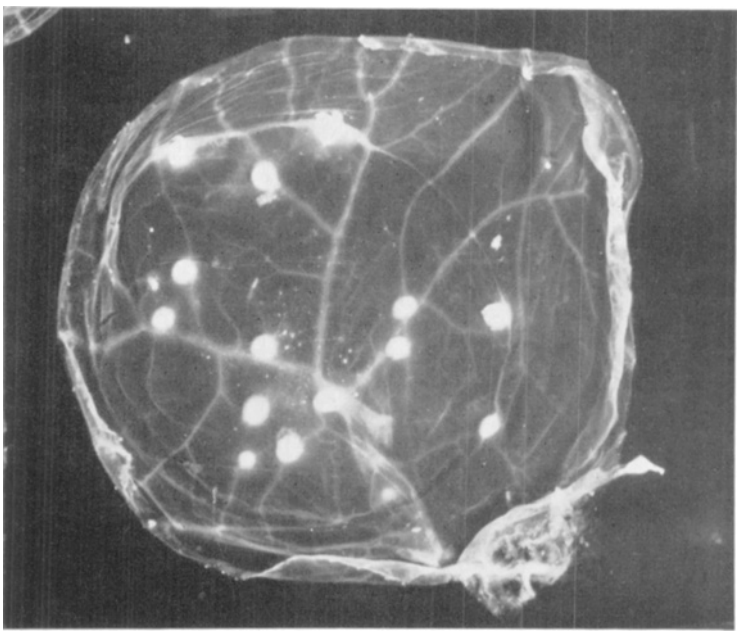

Fig. 6.
Vaccinia virus. Appearance of pocks on the chorio-allantoic membrane (48 hours after inoculation).

VIRUS-ISOLATION

Virus isolation was attempted only from one monkey during outbreak No. 1 and from two monkeys during outbreak No. 2. Virus was recovered from all three animals. The strain isolated first has been most extensively studied but there is no indication of any difference between this strain and those isolated during outbreak No. 2.

Virus isolation in eggs: Scrapings from several papules which were diluted 10^{-3}, 10^{-5}, and 10^{-7} produced greyish oedematous changes in the membranes. In addition small discrete lesions showing a tendency to spread along the blood vessels could be seen in the eggs inoculated with the highest dilution of virus (Fig. 4).

On continued passage, using dilute membrane suspensions as seed, the oedematous reaction disappeared and the small opaque dome-shaped pocks became predominant (Fig. 5). In membranes harvested after incubation for 3 days these pocks resembled closely those described for variola virus. The lesions developed later and were much smaller than those seen with vaccinia virus (Fig. 6). The titer of the original pustule material was 7×10^8 and no significant increase in titer was observed on continued passage in eggs.

Virus isolation in tissue cultures: Pustular scrapings were emulsified

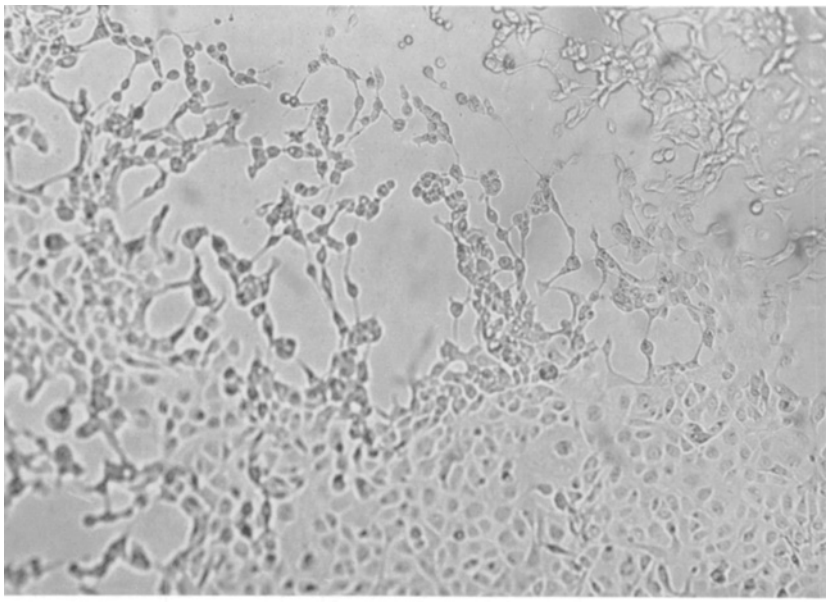

Fig. 7.
Monkey pox virus: Cytopathic changes produced in human amnion cell cultures 2nd tissue culture passage, 5 days after inoculation.

in tryptose-broth, clarified by low speed centrifugation, and the supernatant was inoculated into tissue cultures of monkey kidney, human amnion, and HeLa cells. Cytopathic lesions developed in all cell types after continued incubation for 2–3 days and destruction was almost complete after 5 days.

The cytopathogenic effect was characterized by a rounding of the affected cells which subsequently became granulated and condensed. The cells retained their rounded shape for several days, eventually slipping off the side of the tube leaving macroscopically visible holes in the cell sheet. In the monkey kidney and human amnion cell cultures the affected cells were interconnected by threadlike syncytial elongations (Fig. 7). In the HeLa cell cultures these formations were not observed but otherwise the cytopathogenic effect was similar to that observed in the other cell types used.

On continued tissue culture passage the cytopathogenic changes developed somewhat more slowly, suggesting that the passage fluid contained less virus than the pustular material. This assumption was supported by egg titrations. Tissue culture passage fluid contained only 10^6 to 10^7 pock forming units while the pustular material had a titer of about 10^8. On titration in tissue cultures the titer of passage fluid varied between 10^{-4} and 10^{-6}.

ANTIGENIC RELATIONSHIP OF THE MONKEY-POX AGENT TO VACCINIA VIRUS

The clinical manifestations of the disease suggested strongly that the causative agent might be related to viruses of the variola-vaccinia group. Several experiments were therefore carried out to determine whether the isolated strain was antigenically related to vaccinia virus.

TABLE 1

Neutralization Test on the Chorio-allantoic Membrane.

Virus	Vaccinia Anti Serum		Control (no serum
	Rabbit*)	Human	
Monkey-pox	1.3×10^6	1.8×10^6	15×10^6
Vaccinia	4×10^7	1.2×10^7	10×10^7

*) Hyperimmune sera from rabbits immunized with heat-inactivated antigen.

TABLE 2

Complement-fixation Test:
Antigenic Relationship Between Vaccinia and Monkey-Pox Virus.

Antigen	Dilution of Antigen	Vaccinia-Rabbit-Hyperimmune Serum (England) Dilution of Serum					Control (no serum)
		4	8	16	32	64	
Vaccinia egg	8	+	+	+	+	0	0
	16	+	+	+	+	0	0
	32	+	+	±	±	0	0
	64	+	+	±	±	0	0
	128	+	+	±	±	0	0
	256	+	+	0	0	0	0
	512	+	+	0	0	0	0
Control (no antigen)		0	0	0	0	0	0
Monkey virus egg_2 .	8	+	+	+	±	0	0
	16	+	+	+	0	0	0
	32	+	+	+	0	0	0
	64	+	+	+	0	0	0
	128	±	±	0	0	0	0
	256	±	±	0	0	0	0
	512	±	0	0	0	0	0
Control (no antigen)		0	0	0	0	0	0
Monkey virus TC_2 .	8	+	+	+	±	0	0
	16	+	+	+	0	0	0
	32	+	+	+	0	0	0
	64	+	+	+	0	0	0
	128	±	±	0	0	0	0
	256	±	±	0	0	0	0
	512	±	0	0	0	0	0
Control (no antigen)		0	0	0	0	0	0

Neutralization tests: Human and rabbit vaccinia antisera and a normal rabbit serum were employed in the test. The undiluted sera were mixed with equal amounts of dilute virus suspensions and each mixture was inoculated onto the chorio-allantoic membrane of groups of 5 chick embryos. As can be seen in Table 1 both anti-vaccinia sera neutralized vaccinia and monkey-pox virus to almost the same extent, the reduction of pock-forming units being approximately tenfold.

Complement-fixation tests: The first experiment was carried out using a vaccinia hyperimmune serum received from England against vaccinia and monkey virus antigens. Two antigen preparations of the latter virus were used, one prepared from the 2nd egg passage and one from the 2nd monkey kidney tissue culture passage. The results of a box-titration employing 2 units of complement as shown in Table 2 clearly indicate a relationship between the three antigens examined.

Additional tests were carried out with locally produced rabbit hyperimmune sera against the monkey virus strain. The evaluation of several of these experiments was, however, difficult due to the frequent occurrence of a presumably non-specific reaction between the antigen and sera from normal rabbits. In addition, sera and antigens were frequently of a low titer and had quite often an anticomplementary effect. Nevertheles a definite indication of a relationship between the monkey virus and vaccinia was also obtained with the hyperimmune rabbit sera produced in this laboratory (see Table 3).

TABLE 3

Complement-fixation Test:
Antigenic Relationship between Vaccinia and Monkey-Pox Virus.

Antigen	Dilution of Antigen	Normal Serum (Rabbit 137) Dilution of Serum 8	16	32	64	128	256	Control (no serum)	Monkey Virus Hyperimmune Serum (Rabbit 137) Dilution of Serum 8	16	32	64	128	256	Control (no serum)
Vaccinia egg	8	±	±	±	±	±	±	±	+	+	+	+	+	±	
	16	±	0	0	0	0	0	0	+	+	+	+	±	±	0
	32	0	0	0	0	0	0	0	±	±	±	0	0	0	0
	64	0	0	0	0	0	0	0	±	±	0	0	0	0	0
Contr. (no antig.)		0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monkey virus egg	8	±	0	0	0	0	0	0	+	+	+	0	0	0	0
	16	±	0	0	0	0	0	0	+	+	0	0	0	0	0
	32	±	0	0	0	0	0	0	±	0	0	0	0	0	0
	64	±	0	0	0	0	0	0	±	0	0	0	0	0	0
Contr. (no antig.)		±	0	0	0	0	0	0	±	0	0	0	0	0	0

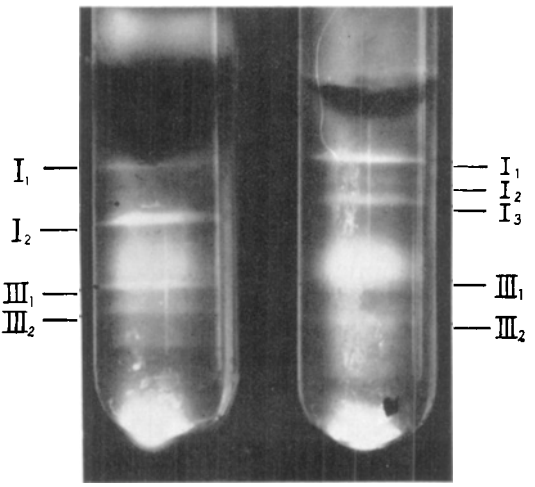

Fig. 8.
Diffusion patterns of vaccinia (right) and monkey pox antigens (left)
Serum: anti-vaccinia.

Hemagglutination-inhibition test: Suspensions of chorio-allantoic membranes infected with either vaccinia or the monkey virus were found to agglutinate chicken red cells to a titer of 1: 60 or more. Erythrocytes from mice were not agglutinated. In tissue cultures infected with either strain no hemaglutinins were detectable.

A hemagglutination-inhibition experiment was carried out using vaccinia virus diluted to contain 4 HA-units against serial twofold dilutions of hyperimmune sera prepared against both viruses. Normal sera from the same rabbits were included as controls in the test. Both immune sera inhibited agglutination of vaccinia virus hemagglutinin to the same extent (serum titer 1: 768).

Diffusion-precipitation test: A diffusion-precipitation test was carried out testing the monkey agent and vaccinia virus against an anti-vaccinia rabbit serum (Fig. 8).

The precipitation pattern may be divided into two major fractions I and III (12) and each of these again into minor zones. With vaccinia virus (right tube) fraction I consists of three zones: a distinct upper zone I_1, a weak zone I_2, and a zone I_3 almost as distinct as I_1. With the monkey virus (left tube) fraction I consists of only two zones: a weak upper zone I_1, and a distinct lower zone I_2. The distance between these zones is broader than is the distance between I_1 and I_3 for vaccinia. The upper precipitation zone probably corresponds to Gispen's fraction I which according to this author is lacking when cow-pox virus is tested against vaccinia antiserum (12). The monkey agent thus seems to be antigenically different from vaccinia as well as from cow-pox virus.

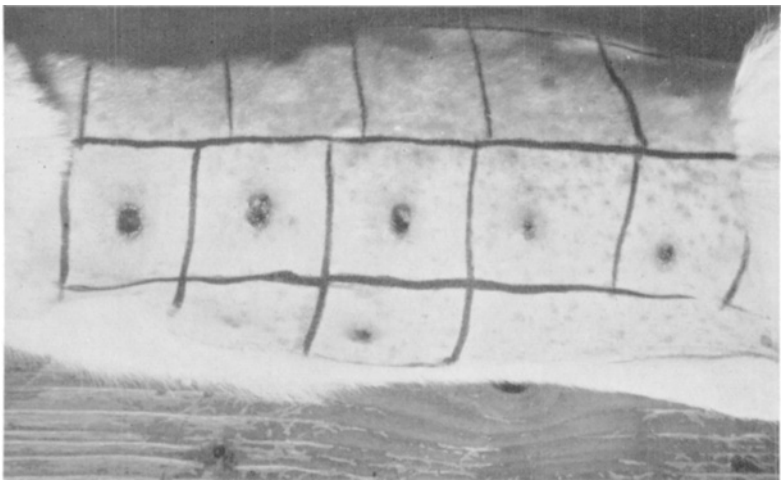

Fig. 9.
Rabbit 948, left side: *Monkey pox virus lesions in the skin of a rabbit:* Squares in the middle row from left to right inoculated with egg passage virus diluted 1: 100, 1: 1000, 1: 3000, 1: 9000, and 1: 27,000; two lower squares inoculated with virus diluted 1: 81,000 and 1: 243,000, respectively.

ATTEMPTS TO DEMONSTRATE A RELATIONSHIP OF THE MONKEY VIRUS TO HERPES SIMPLEX AND TO B-VIRUS

A neutralization test using a known herpes immune serum and 100 TCD_{50} of monkey-pox virus was carried out in HeLa cell cultures. There was no evidence of neutralization of the monkey agent by herpes hyperimmune serum.

In a study which was kindly performed at the virus Reference Laboratory, London, it was shown that an immune B-virus guinea-pig serum had no neutralizing effect on the monkey agent (F. O. MacCallum, personal communication).

SUSCEPTIBILITY OF LABORATORY ANIMALS TO THE MONKEY VIRUS

Adult rabbits: Intradermal inoculation: Depilated rabbits were inoculated with 0.1 ml amounts of serial dilutions of monkey virus. The severe hemorrhagic reactions which developed were very different from those caused by vaccinia virus. A 10^{-5} dilution of egg passage virus with a pock count titer of 8×10^7 caused necrosis 10 mm in diameter (see Figs. 9 and 10).

Inoculation by scarification: Egg passage monkey virus diluted 1: 1000 produced a confluent lesion when rubbed into a scarified area 5 cm^2 in size. Single pustules developed following inoculation of 1: 80,000 diluted virus. The lesions were smaller and developed later

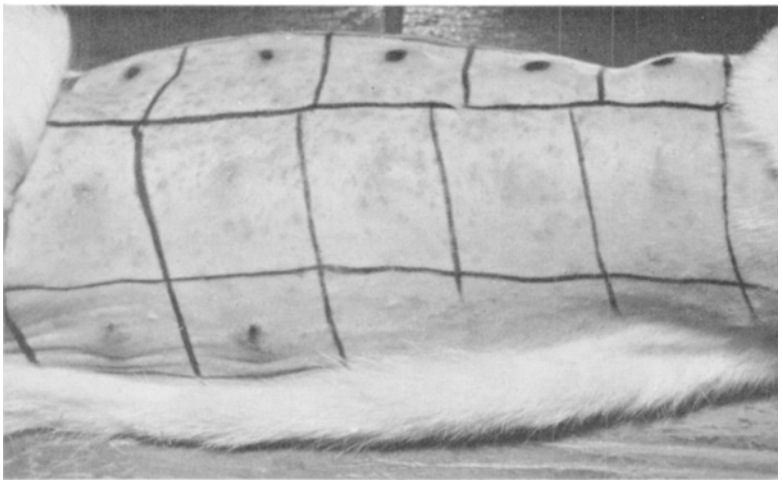

Fig. 10.
Rabbit 948, right side: *Vaccinia virus lesions in the skin of a rabbit.* The lower square right virus diluted 1:100, left virus 1: 1000. Middle row of squares from left to right with virus diluted in 3 fold steps from 1: 3000 to 1: 243,000. Reactions produced by monkey pox virus seen in upper row of squares (from right to left virus diluted in 3 folds steps 1: 3000 to 1: 243,000).

than in animals infected with vaccinia virus by the same route. Serial passages of monkey virus by scarification were successful and there was no indication of a decrease in titer on passage. Several observations indicate, however, that the monkey virus has a greater affinity for the subdermal than for the dermal epithelium.

Re-isolation of monkey virus from rabbits: Virus was recovered from the 2nd rabbit skin passage in tissue cultures and on the chorio-allantoic membrane. The cytopathogenic effect as well as the pocks were indistinguishable from those observed during the original isolation.

Generalized infection in rabbits was observed in a number of instances 5–6 days after inoculation by scarification or by the intracutaneous or intravenous routes. At autopsy about 1 month later when the animals had fully recovered no lesions were observed in their organs.

Active protection test: Rabbits which had recovered from infection by scarification with either vaccinia or monkey virus showed the same degree of immunity to a subsequent infection by the same route with either virus. In animals challenged 4 weeks after the initial exposure a reduction in titer from 80,000 to 1000 was thus observed for vaccinia and monkey virus, respectively.

Infection of 2 day old rabbits: Vaccinia and monkey virus both caused a fatal infection in 2 day old rabbits. Following inoculation by scarification or by the intra-cutaneous route of 10 per cent suspensions of vaccinia-infected egg membranes death occurred after 4 to 6 days. With monkey virus material death was delayed for a further 1 to 2 days. Both viruses caused earlier death of the animals when given by

the intracutaneous route. The two viruses induced the same pathological changes, *i.e.* greyish-white spots in the liver tissue and hemorrhagic foci in the kidneys, as described earlier by Ørskov and Andersen, for vaccinia (27).

Corneal inoculation of rabbits: A suspension of chorio-allantoic membranes infected with the 3rd egg-passage of the monkey agent was applied to the scarified cornea of a rabbit. The animal was sacrificed 3 days later and the eyes removed and treated with corrosive sublimate. The cornea of the inoculated eye showed 4–5 discrete white opaque elevations with a central crater.

Adult mice: Intracerebral inoculation of adult white mice with egg passage material of the monkey agent diluted 10^{-4} or less caused encephalitis and subsequent death of all the animals. Serial passage by the intracerebral route was successful with brain material diluted 10^{-2}. Altogether 3 intracerebral passages were carried out. On each passage level all mice inoculated with 10^{-2} diluted material succumbed to the infection. Virus was recovered on the chorio-allantoic membrane from 10^{-3} diluted brain tissue of the 3rd mouse passage.

Suckling mice, two days old were inoculated intranasally by a method described earlier (26). Egg passage material of both viruses diluted 10^{-4} killed 100 per cent of the inoculated animals. Death occurred 5 to 14 days after inoculation of the monkey agent and somewhat earlier after infection with vaccinia virus, *i.e.* 5 to 8 days after inoculation (see Table 4).

TABLE 4

Intranasal Infection of Suckling Mice with Vaccinia and Monkey Virus.

Virus Strain	Dilution of Egg-virus			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Monkey virus	3/3* (5)†	4/4 (6–7)	5/5 (8–14)	0/5
Vaccinia	4/4 (5–7)	4/4 (4–9)	4/4 (7)	2/5 (7–8)

*) Nominator: number of mice died. Denominator: number of mice inoculated.
†) Figures in paranthesis record day of death.

Monkeys: Two Rhesus monkeys were inoculated intradermally in the palm of the hand with 0.2 ml of tissue culture material. None of these animals developed any signs of illness. However, one cynomolgus monkey belonging to a shipment not affected by the disease, on similar inoculation developed a local pustule surrounded by oedema 7 days after inoculation. This animal's temperature was slightly elevated between the 5th and 9th day after inoculation but no spread of the eruption occurred. Attempts to re-isolate virus from the lesions were unsuccessful because of heavy contamination with molds and bacteria.

Inoculation of other laboratory animals: Guinea pigs and chickens were used for production of hyperimmune sera. They were inoculated

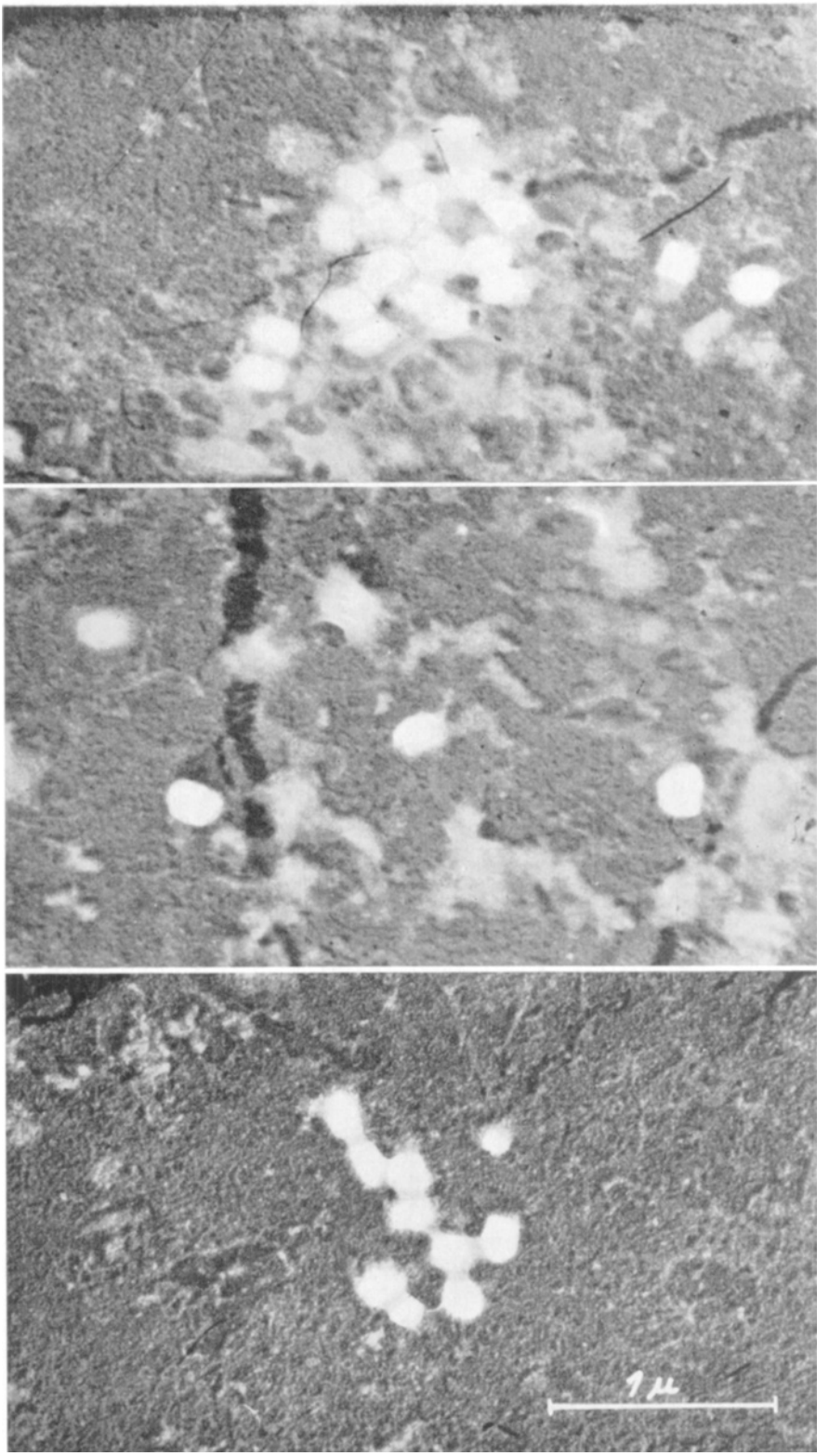

Fig. 11.

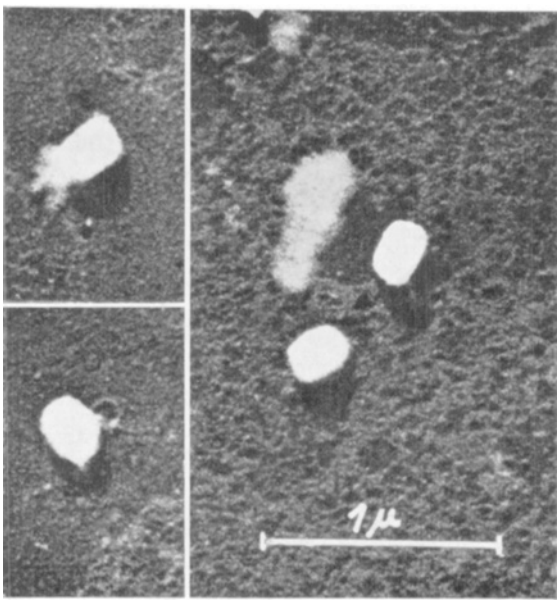

Fig. 12.

Figs. 11 and 12.
Electron micrographs of monkey pox virus:
Fig. 11. Virus particles in pus-like material from a pustular lesion of a monkey. Platinum shadowcast. Magnification approximately 30,000 ×.
Fig. 12. Virus particles from a partially purified suspension of the 3rd egg passage. Platinum shadowcast. Magnification approximately 30,000 ×.

intravenously, intraperitoneally as well as subcutaneously with egg passage and tissue culture passage material of the monkey virus. None of these animals showed any signs of disease.

RESISTANCE TO STORAGE, ETHER, AND FORMALDEHYDE

Storage: The monkey-pox virus retained its infectivity in the lyophilized state or at —60° C for several months. Suspensions of infected membranes or of tissue culture fluid showed a gradual decrease in infectivity when kept at + 4° C for several weeks. A considerable loss in titer occurred in suspensions stored at —15° C for one week.

Ether: Exposure of approximately $10^{4.5}$ tissue culture infective doses of the agent to 20 per cent anesthetic ether overnight in the cold (2) did not influence the infectivity.

Formaldehyde: Treatment of approximately $10^{4.0}$ tissue culture infective doses with formaldehyde 1: 4000 at 37° C resulted in a gradual loss in infectivity and after approximately 50 hours no infectious virus could be detected on subculture in monkey kidney cell cultures.

PRESENCE OF MONKEY-POX VIRUS IN TISSUE CULTURES PREPARED FROM KIDNEYS OF APPARENTLY HEALTHY MONKEYS

During the first outbreak of the pox disease some apparently healthy cynomolgus monkeys which belonged to the affected shipment had been sacrificed for other purposes. The kidneys from these animals were trypsinized and culture tubes were prepared from the cell suspensions. In some of the tubes (approximately 5 per cent) cytopathic changes developed 7 to 12 days later and monkey-pox virus was isolated from these cultures.

During the 2nd outbreak monkey-pox virus was again present in a small percentage of the tubes prepared from kidneys of apparently healthy—but exposed—animals. On this occasion virus was recovered from cultures of a monkey which had been sacrificed 4 days before the outbreak. This was the only occasion when monkey-pox virus was encountered in cultures which had been prepared at a time when clinical illness was not present in the colony. In all instances the cytopathic manifestations in the cultures were not recognisable until 7 to 10 days after the tubes had been prepared indicating that only small amounts of virus had been present in the cell stock suspension.

ELECTRON MICROSCOPY

The results obtained by electronmicroscopy are presented in Figs. 11 and 12.

Fig. 11 shows particles obtained directly from the pustular lesions of a monkey, whereas the elementary bodies of the virus obtained from the third passage on chorio-allantoic membranes of material isolated from another monkey are given in Fig. 12. Although the fields presented are not particularly well free of accompanying impurities they do bring out the outlines of quite a few particles clearly enough to allow the well known rectangular appearance characterizing the viruses of the pox-group to be distinguished. In a few cases the dimensions of well isolated particles have been measured. They all fell within the range of the pox viruses, *i.e.* 200 by 250 mμ. Especially one of the clusters of particles shown in Fig. 11 appears somewhat smaller, but this is probably the result of shrinkage during the preparation. Isolated particles of both types of material generally measure approximately 200–250 mμ as stated above.

DISCUSSION

Natural pox diseases are known to occur in a variety of animal species but to our knowledge only one outbreak of spontaneous pox in monkeys has been described before. This outbreak occurred in Brazil

during a mild epidemic of small-pox among the natives in Alto Uruguay. The disease spread to Mycetes- and Cebus monkeys which developed typical pustules and died in large numbers. The etiologic agent was, however, not recovered from the infected animals (3).

The present study reports 2 outbreaks of a non-fatal pox-like disease in cynomolgus monkeys housed in this institute. The clinical manifestations observed in the monkeys were so typical that it seemed fairly certain that the disease was caused by a pox virus. This assumption was substantiated by the isolation from the pustules of an agent which grew readily on the chorio-allantois of chick embryos producing characteristic focal lesions. Morphologically the agent was found to have the size and brick-shaped appearance typical for members of the pox virus group. Furthermore, an antigenic relationship between the newly isolated agent and vaccinia virus could be demonstrated by neutralization-, hemagglutination-inhibition-, and complement-fixation tests as well as by active protection tests in rabbits.

A number of these observations were confirmed in a study which kindly was carried out in the Virus Reference Laboratory, London. It was found that when the 3rd monkey kidney tissue culture passage fluid from Copenhagen was inoculated onto the chorio-allantoic membrane of 10–12 day old fertile eggs it produced lesions in 72 hours which were indistinguishable from variola, and the virus was almost completely neutralized by vaccinia immune rabbit serum. (F.O.MacCallum, personal communication).

Extended studies in our laboratory on the relationship of the monkey virus to known pox-viruses have so far not led to conclusive results. The appearance of the lesions on the chorio-allantois resembled —as mentioned above—closely variola (7). The lesions produced by such egg-passage material of the monkey agent on the scarified cornea of a rabbit were also closely similar to those described for small-pox (19). However, the monkey virus differed from small-pox virus in two important characteristics. It could be passed continuously in adult white mice by the intracerebral route and it was readily maintained in serial cutaneous passages in the rabbit skin (23). It may of course be argued that the monkeys were originally infected with variola and that the virus by its passage in monkeys had achieved the ability of multiplying in the central nervous system of mice as well as in the rabbit skin. This possibility cannot be excluded since it has been shown by several workers that passage of variola through a monkey may assist in securing subsequent transfer to other animals. It should, however, be emphasized that neither on intracerebral passage in mice nor on serial transfer in the rabbit skin was any evidence obtained that a transformation of the monkey pox agent into vaccinia (Pox virus officinale (10)) had occurred. In both instances the strain retained its ability to produce small variola-like pocks on the chorio-allantois.

There is no evidence indicating that the monkey virus may be an

ectromelia virus, since it did not cause any disease in intraperitoneally or intradermally inoculated adult mice and it did not agglutinate mouse erythrocytes (4).

Finally, the monkey agent could not be identified as a strain of cow-pox virus (Pox virus bovis (10)). Although the hemorrhagic lesions in the rabbit skin produced by the monkey virus appeared similar to those described for cow-pox (6, 9), the chorio-allantoic lesions were quite distinct from those typical for cow-pox (6, 9, 13). The possibility that the monkey agent might represent a white-variant strain of cow-pox which produces fairly small non-hemorrhagic foci in the chorio-allantois can probably also be disregarded since this variant in its pure form will not cause hemorrhagic lesions in the rabbit skin (8). Finally, attempts to identify the monkey agent as cow-pox virus by the precipitation-diffusion test (12) were unsuccessful.

At present, therefore, the monkey-pox agent cannot be classified with any of the well established pox viruses, and the possibility that the monkey virus represents a separate entity cannot be excluded.

Concerning the epidemiology of the disease, it seems unlikely that the virus was introduced into the monkey colony after the animals had arrived in this country. No cases of human small-pox have occurred in this country for more than 25 years, and pox diseases in other mammals are likewise uncommon in Denmark. Cow-pox is seen occasionally but no cattle are kept on the grounds or in the vicinity of this institute. Ectromelia was present in the laboratory stock of white mice some ten years ago but since then no indication of the presence of this virus has been encountered. Rabbit-pox is not known to have occurred at any time. The improbability that the virus infection originated in this country is further strengthened by the quarantine under which the monkeys were held, making any contact with other animals very unlikely.

However, the long period of time which elapsed between the arrival of the monkeys in the laboratory and the outbreaks of the disease, *i.e.* 51 and 62 days, respectively, is rather puzzling and can hardly be attributed to an incubation period of this length. In the experimentally infected monkeys the pox lesions appeared within 7 days after inoculation indicating an incubation period similar to that observed in experimental infections of monkeys with vaccinia or small-pox virus (16).

The most likely explanation of the long latent period is that the virus was present on arrival in some of the animals as a silent infection. This is, however, the only evidence to suggest that long term latency can occur. The fact that the pox virus was isolated from kidney cell cultures prepared from apparently healthy monkeys during and shortly before the outbreak of manifest disease may only indicate that virus can be present in the animals before onset of clinical disease during an initial viremic phase.

Although it cannot be definitely excluded that outbreak No. 2 was due

to infection with virus surviving from the first outbreak such an explanation seems unlikely in view of the fact that the second outbreak began in an animal room which is located in a separate building at least 200 yards from the main building to which outbreak No. 1 was limited.

In conclusion, it seems most likely that both shipments of cynomolgus monkeys harboured the pox-virus on their arrival in this laboratory.

Virus diseases in monkeys living under natural conditions have certain implications of practical epidemiological importance since such animals may serve as reservoirs for viruses pathogenic for other animals and for human beings. From a laboratory point of view, the presence of virus infections in monkey colonies poses a considerable problem because of the increased use of these animals in recent years for virus research and for polio vaccine production. Viruses present in the kidneys may contaminate the cell cultures prepared from these organs, and are, therefore, of particular importance. Although the pox-virus described in this paper was observed in normal tissue cultures prepared from kidney tissue, this fortunately happened only during short periods of time and the virus concentration was apparently low. Studies on the inactivation by formaldehyde of the monkey virus indicated that a possible contamination with this virus strain will probably not incriminate the safety of polio vaccine prepared from such monkey kidney tissue cultures.

SUMMARY

(1) Two outbreaks of a spontaneous pox disease in the monkey colony in this institute are described. Both outbreaks occurred late after arrival of the monkeys, *i.e.* after 51 and 62 days, respectively, and only 20 to 30 per cent of the animals developed clinical signs of disease.

(2) The clinical manifestations were characterized by skin eruptions of a maculo-papular rash and of variolous pustules. The general health of the animals was not seriously affected and fatal cases were not observed.

(3) A virus was isolated from the pustular lesions. This agent multiplied on the chorio-allantoic membranes and produced cytopathic changes in tissue cultures of monkey kidney-, human amnion-, and HeLa cells. The monkey virus was serologically related to vaccinia and had the brick-shaped appearance typical for pox-viruses.

(4) The monkey virus caused variola-like lesions on the chorio-allantois and on the scarified cornea of rabbits. However, unlike variola it could be maintained in serial passages in mice by the intracerebral route causing encephalitis and in the rabbit skin where it produced severe hemorrhagic lesions. It caused a fatal infection in 2 day old rabbits inducing typical pathological changes in the liver and in the

kidneys. Attempts to identify the monkey virus with any of the known pox-viruses failed.

(5) A small percentage of tissue culture tubes prepared from apparently healthy monkeys which had been sacrificed during the two outbreaks were found to be contaminated with the monkey pox virus.

The observations on the presence of monkey pox virus in the normal tissue cultures as well as some of the studies on intracerebral infection of adult mice and the formaldehyde-inactivation experiments were made in the polio department of this institute. We are indebted to dr. *Annelise Godtfredsen* and dr. *Inger Petersen* for permission to publish their results. Our thanks are also due to dr. *Sven Tulinius*, department of epidemiology, for his kind advice and assistance.

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